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PRELIMINARY STUDY ON EFFECTS OF UNREFINED VIBRIO TOXINS ON SURVIVAL OF AND HEMOLYMPH COAGULATION IN WHITE SHRIMP, Penaeus setiferus[1/]

J. K. Leong and D. S. Hanrahan
U.S. Department of Commerce
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Southeast Fisheries Center
Galveston Laboratory
4700 Avenue U
Galveston, Texas 77550

ABSTRACT

Intramuscular injection of a 0.05-ml aliquot of crude endotoxin (CREND) or exotoxin (CREXT) extracts of Vibrio parahemolyticus or V. alginolyticus induced significant mortalities in juvenile white shrimp (Penaeus setiferus). In normal white shrimp, the hemolymph becomes viscous within 30 seconds and coagulated within one minute after withdrawal from the body. In CREND-treated shrimp, viscosity and coagulation may be delayed up to almost one hour and four hours, respectively, or may not occur at all. This anticoagulation phenomenon is contrary to the intravascular coagulation effect of endotoxin in mammals. It also differs from the in vitro enhancement of gelation of Limulus blood by endotoxin.

INTRODUCTION

Previous experiments indicated that a toxic factor or factors were involved in the death of penaeid shrimp infected with Vibrio bacteria (Leong and Fontaine, 1979). It will be of practical interest to determine the mode of toxigenesis in the shrimp. As a first step to probe into this question, preliminary experiments were conducted to test on juvenile white shrimp, Penaeus setiferus, crude toxic fractions prepared from Vibrio parahemolyticus and V. alginolyticus.

The procedure for obtaining crude toxic fractions was similar to that which was used to prepare crude bacterial endotoxin and exotoxin extracts. For immediate purposes, we did not further refine the extracts. Refinement work was projected to be done in a second phase

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of the research, which will be carried out at another time when circumstances permit. For convenience sake, we shall refer to the crude extracts as crude endotoxin, (CREND) and crude exotoxin (CREXT) in this paper.

MATERIALS AND METHODS

Juvenile white shrimp, 62-138 mm long, were maintained individually in glass aquaria converted from gallon-sized Mason jars (Leong and Fontaine, 1979). The animals were injected intramuscularly, according to a procedure adopted by Lightner and Lewis (1975), between the 4th and 5th abdominal segments with a 0.05-ml aliquot of a crude preparation of either CREND or CREXT obtained from V. parahemolyticus (strain GFC-76-36) isolated from postlarval white shrimp (Penaeus setiferus) in Galveston Laboratory, National Marine Fisheries Service, and identified by Dr. D. H. Lewis, Texas A&M University, College Station, Texas, and V. aliginolyticus (strain MS-670; NMFS culture code GFC-76-5) obtained from Dr. D. H. Lewis, Texas A&M University. These bacteria had been grown for 24 hr in brain-heart infusion medium (BHI).

CREXT was prepared by centrifugation (1,500 g, 10 min) of the bacteria from a BHI culture and passing the supernatant through a disposable Nalgene filter (0.2 micron) (Nalgene Co., New York[2]) seated in an ice-bath. The filtrate if not immediately used was frozen at -20°C and quickly thawed just before use.

To prepare CREND, the above bacterial pellet was resuspended in a sterile saline solution (2.5% NaCl) to the original volume and centrifuged. The washed cells were again resuspended in a similar saline solution to the original culture volume. The suspension was then steam-atuoclaved (121°C, 15 psi, 15 min) to lyse the bacterial cells, to be followed by freezing and storage at -20°C.

Groups of 5 shrimp injected with different dilutions of either CREND or CREXT (Tables 1 and 2) were periodically monitored for mortality, while only shrimp injected with CREND were monitored for changes in hemolymph. Control groups consisted of shrimp receiving or not receiving injections of BHI or saline. To monitor hemolymph changes, a sterile hypodermic needle (gauge 27) fitted to a 1 c.c. tuberculine syringe was inserted dorsally into the pericardial sinus. The withdrawn hemolymph was expelled from the syringe (needle removed) into the cavity of a depression slide and left at room temperature (23°C). Periodic records were made of the viscosity and color of the hemolymph.

[2]Mention of trade names or commercial products does not constitute endorsement for use.

RESULTS AND DISCUSSION

Both CREND and CREXT from the two Vibrio species were highly toxic to P. setiferus via intramuscular injection (Figs. 1, 2). The undiluted toxin preparations yielded very steep mortality curve within 24 hr, indicating acute intoxication reaction in animals. These results confirmed the toxic effect of the crude extracts on the shrimp.

Vibrio bacteria are common in shrimp rearing tanks and can multiply rapidly in seawater containing a high level of organic nutrients. These bacteria release toxins during their life processes. How the presence of free bacterial toxins in the tank water affect the survival of the shrimp is unknown. There is evidence that shrimp food contaminated with Vibrio bacteria could cause death in penaeid shrimp, presumably due to the effect of bacterial toxins rather than direct invasion by the bacteria (Leong and Fontaine, 1979). In laboratory tests, a species of Pseudomonas (GFC-80-9), which was isolated from seawater used in culturing larvae of penaeid shrimp, Penaeus stylirostris, was observed to be lethal to naupliar and protozoeal stages of shrimp larvae (J. K. Leong, unpublished data). Since there was no apparent tissue invasion by the bacteria, it was postulated that the lethal effect of GFC-80-9 was mediated through bacterium-associated toxins. This toxin-mediated-mortality concept was later shown to be valid by laboratory experimental findings (C.L. Huang, personal communication). In controlled experiments, Huang placed endotoxin, chemically extracted from Pseudomonas GFC-80-9, in seawater containing either naupliar or protozeal larvae of P. stylirostris or newly-hatched larvae of brine shrimp, Artemia salina, and found that the intoxicated culture water was lethal to the shrimp larvae. These observations and the fatal effects of CREND and CREXT in shrimp as demonstrated in the present study have implied the vulnerability of penaeid shrimp to bacterial toxins in culture water.

In normal white shrimp, the hemolymph became viscous within 30 seconds and coagulated within one minute after withdrawal from the body (Tables 1-3). In CREND-treated shrimp, viscosity and coagulation could be delayed up to 1 hr and 4 hr, respectively, or might not occur at all. This anticoagulation phenomenon is in contrast with the intravascular coagulation effect of endotoxin, which constitutes the Schwartzman reaction, in man and rabbits (Braude et al., 1973; Jawetz et al , 1972; Smith et al., 1964).

In experiments using live Vibrio bacteria instead of bacterial toxic extract as inoculum, Lightner and Lewis (1975) observed that hemolymph drawn from bacterium-infected moribund shrimp could not clot as rapidly as hemolymph taken from uninoculated shrimp, and that Giemsa-stained hemolymph smears from moribund shrimp contained hemocytes in greatly reduced numbers compared to normal shrimp. These

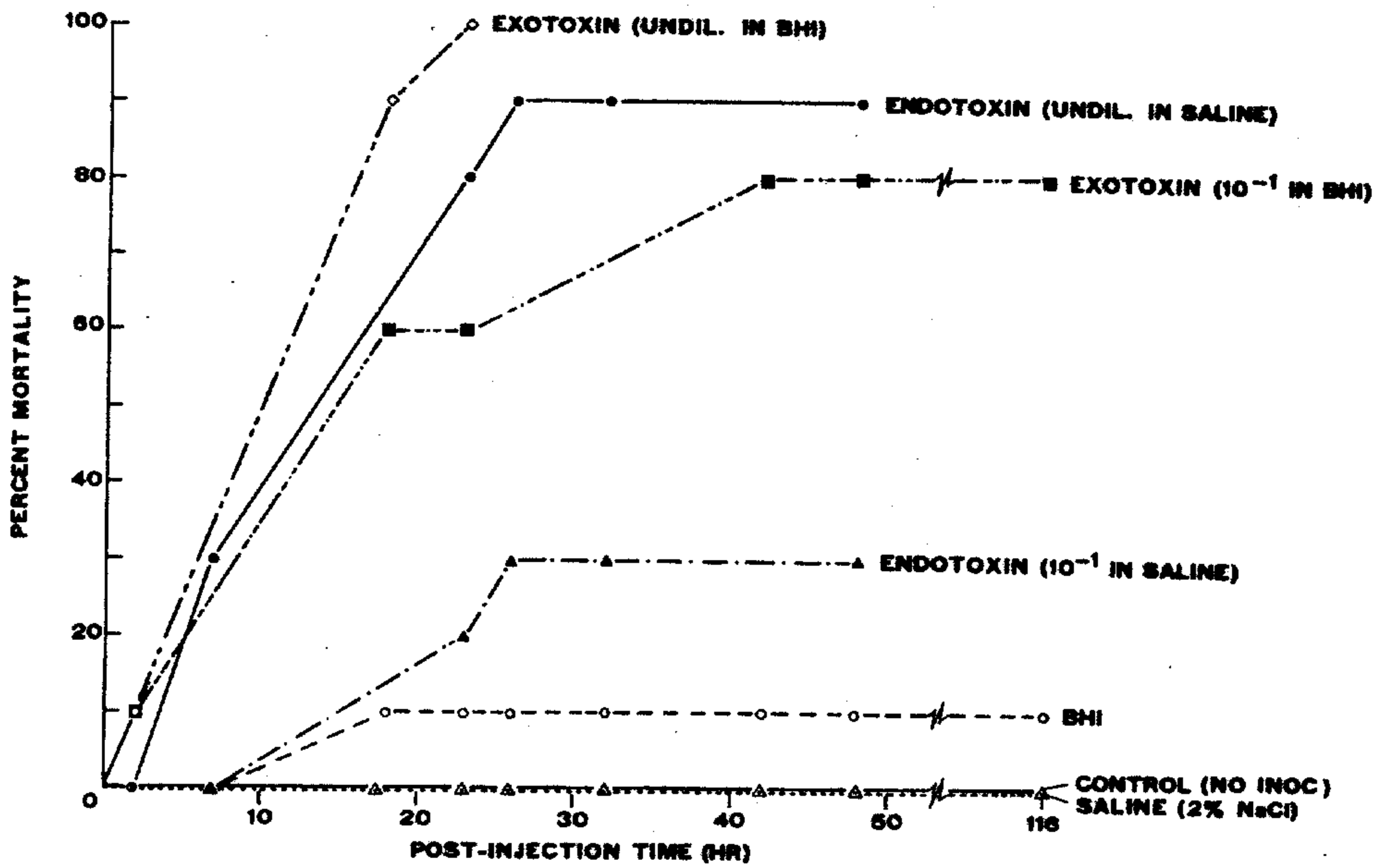

Figure 1. Mortality of Penaeus setiferus injected with crude endotoxin and exotoxin extracts of Vibrio parahemolyticus. Undil., undiluted; BHI, brain-heart infusion broth; Inoc., inoculum.

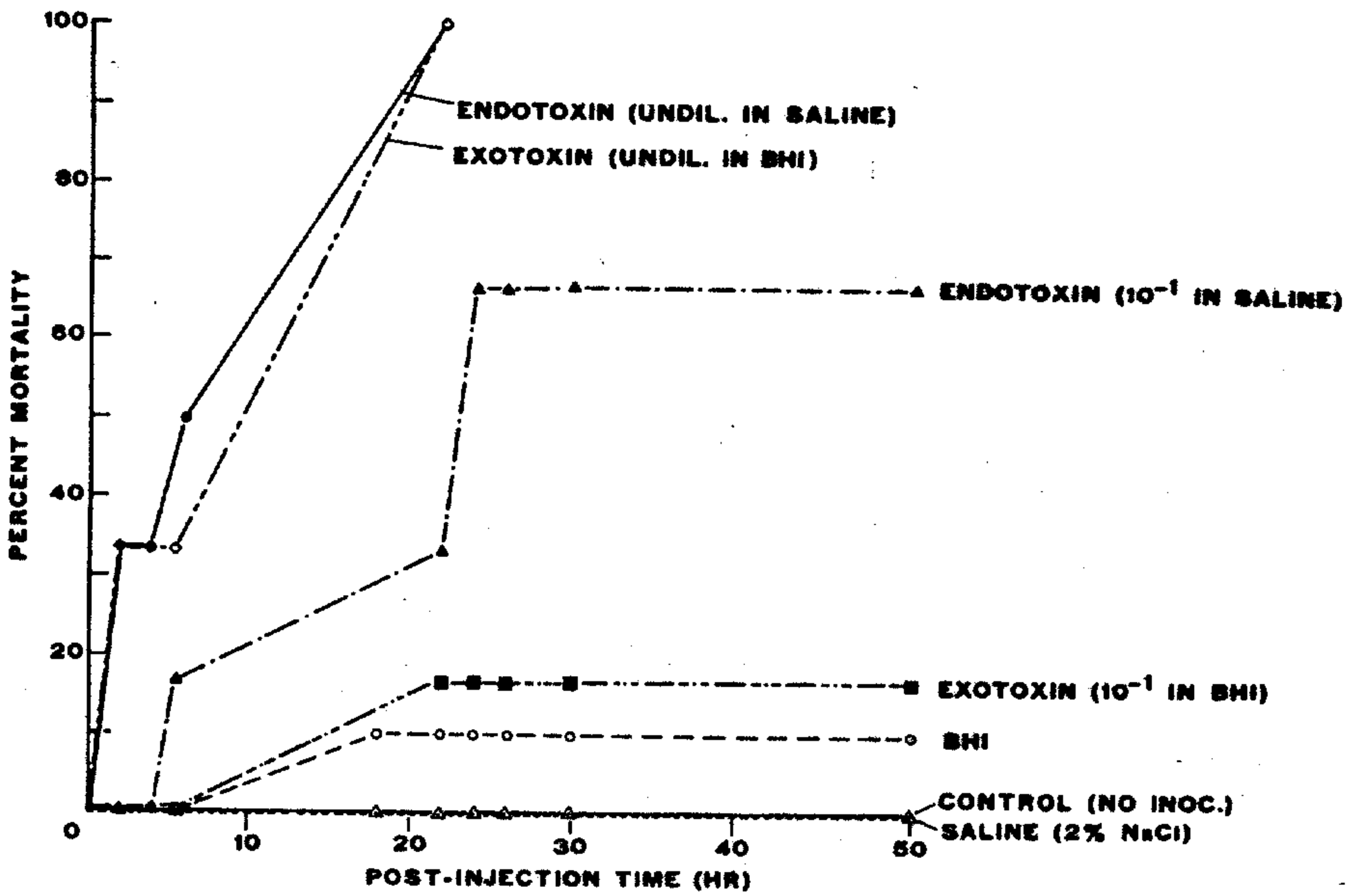

Figure 2. Mortality of Penaeus setiferus injected with crude endotoxin and exotoxin extracts of Vibrio alginolyticus. Undil., undiluted; BHI, brain-heart infusion broth; Inoc., inoculum.

Table 1. Changes in physical conditions of hemolymph withdrawn from white shrimp (Penaeus setiferus) one hour after injection of shrimp with unrefined endotoxin from Vibrio alginolyticus.

Inoculum	No. of Shrimp Tested	A Time (min) for Hemolymph Changes To Viscous	B To Gelatinous	C Hemolymph Color When Drawn	D Condition of Shrimp	Shrimp Showing Conditions in Columns A - D No.	%
Endotoxin (undil.)	5	7	15	LB[a]	Weak	2	40
		14	17	LB	Weak	1	20
		7	225	TBC[b]	Weak	1	20
		NC[c]	NC(240)[d,e]	TBC	Weak	1	20
Endotoxin (10^{-1} dil.)	5	Inst (vv)[f]	1	LB	Normal	2	40
		Inst (sv)[g]	8	LB	Normal	3	60
Saline (2.5% NaCl)	5	Inst (vv)	1	LB	Normal	5	100
None (Control)	5	Inst (vv)	1	LB	Normal	5	100

[a]Light blue
[b]Trace of blue in a colorless background
[c]Not changed
[d]Not changed after 240 minutes
[e]Hemolymph remained fluid after further storage for 2 days at 4°C.
[f]Very viscous instantly (within 30 seconds)
[g]Slightly viscous instantly (within 30 seconds)

Table 2. Changes in physical conditions of hemolymph withdrawn from white shrimp (Penaeus setiferus) 5 hours after injection of shrimp with unrefined endotoxin from Vibrio alginolyticus.

Inoculum	No. of Shrimp Tested	A Time (min) for Hemolymph Changes To Viscous	B To Gelatinous	C Hemolymph Color When Drawn	D Condition of Shrimp	Shrimp Showing Conditions in Columns A - D No.	%
Endotoxin (undil.)	5	NC[a]	60	TBC[b]	Very weak	1	20
		SV (60)[c]	NC (240)[d]	TBC	Weak	1	20
		NC	NC (240)	LB[e]	Very weak	2	40
		(Shrimp died; hemol. not examined)			Dead	1	20
Endotoxin (10^{-1} dil.)	0	-	-	-	-	-	-
Saline (2.5% NaCl)	5	Inst (vv)[f]	1	LB	Normal	5	100
None (Control)	5	Inst (vv)	1	LB	Normal	5	100

[a]Not changed
[b]Trace of blue in a colorless background
[c]Slightly viscous within 60 minutes
[d]Not changed after 240 minutes
[e]Light blue
[f]Very viscous instantly (within 30 seconds)

Table 3. Changes in phvsical conditions of hemolymph withdrawn from white shrimp (Penaeus setiferus) 24 hours after injection of shrimp with unrefined endotoxin from Vibrio alginolyticus.

Inoculum	No. of Shrimp Tested	A Time (min) for Hemolymph Changes To Viscous	B To Gelatinous	C Hemolymph Color When Drawn	D Condition of Shrimp	Shrimp Showing Conditions in Columns A - D No.	%
Endotoxin (undil.)	0	-	-	-	-	-	-
Endotoxin (10^{-1} dil.)	5	NC[a]	3	TBC[b]	Normal	1	20
		NC	NC (360)[c]	TBC	Normal	1	20
		NC	NC (360)	TBC	Very weak	1	20
		(Shrimp died; hemol. not examined)		-	-	2	40
Saline (2.5% NaCl)	5	Inst (vv)[d]	1	LB[e]	Normal	5	100
None (Control)	5	Inst (vv)	1	LB	Normal	5	100

[a]Not changed
[b]Trace of blue in a colorless background
[c]Not changed after 360 minutes
[d]Very viscuous instantly (within 30 seconds)
[e]Light blue

observations coupled with the findings in our study have suggested that morbidity or death of shrimp in Vibrio infection might be due to non-coagulation of shrimp hemolymph, which could have been caused by bacterium-associated toxins such as endotoxin and exotoxin. The reduction in shrimp hemocytes as observed by Lightner and Lewis (1975) could have been caused by either invasion of bacteria or direct effects of bacterial toxins. Unfortunately, attempts to assess quantitatively the possible influence of CREND on destruction of shrimp hemocytes in our study were unsuccessful, primarily due to a great variation of hemocyte counts in different shrimp.

A different kind of response of crustacean blood to bacterial endotoxin had been described by Levin and Bang (1968) in Limulus, the horseshoe crab. Like shrimp blood, the whole blood of Limulus, after withdrawal, would quickly form a cellular clot. This clot contained aggregated amebocytes which later would degenerate and produce a liquid phase designated as pre-gel. The disruption of amebocytes would enhance the production of pre-gel. When pre-gel was exposed to bacterial endotoxin, coagulation occurred, and the rate of coagulation was directly related to the rate of concentration of endotoxin. Cell-free Limulus plasma was incoagulable. Disodium ethylenediamine teraacetate (EDTA) blocked the gelation effect of endotoxin. The sensitivity of the Limulus reaction has been well recognized and employed by health scientists for the detection of endotoxemia in Gram-negative sepsis (Levin et al., 1972; Wildfeuer et al., 1974).

Why CREND induced non-coagulation of shrimp blood instead of coagulation such as what endotoxin did to Limulus blood has not been understood. It could have been a matter of procedural differences in experimentation such as in vivo test in shrimp versus in vitro for Limulus blood. Nevertheless, the anticoagulation characteristic of Vibrio endotoxin in penaeid shrimp could be highly significant. It may facilitate studies on the mechanism of pathogenesis on vibriosis and the mechanism of hemolymph coagulation in shrimp. The underlying cause for hemolymph coagulation in shrimp is not well understood. It is difficult to study and analyze shrimp blood because it gels rapidly after leaving the body.

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